INHIBITION OF FERTILIZIN AGGLUTINATION AND FERTILIZATION IN ARBACIA BY FUCUS EXTRACTS[1]

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A number of substances which impair or inhibit the normal fertilization response of echinoderm gametes have been investigated. These include natural substances from the gametes themselves (reviewed in Metz, 1957), fluids from the parent animals (Metz, 1960), and various extraneous agents (Wicklund, 1954a). Of these various agents, those which inhibit fertilization without "injuring" the unfertilized egg or spermatozoa are of most interest (Wicklund, 1954a). Harding (1951) reported that crude "fucoidin," a polysaccharide from the brown alga *Fucus vesiculosus*, was such an inhibitor. That observation led to a more detailed investigation of the fertilization inhibitor from *Fucus*, designated FeInh(Fu) by the Swedish investigators (Wicklund, 1954c; Esping, 1957a, 1957b; Runnström *et al.*, 1959) and Branham and Metz (1959, 1960).

Wicklund (1954a) demonstrated that sea urchin spermatozoa were not paralyzed by FeInh(Fu) solutions and were capable of fertilizing eggs after an exposure to the extracts of up to eight hours. The same investigator also found the inhibitory effects on eggs to be reversible when the material was washed from the eggs. It was concluded that the FeInh(Fu) had to be present at the time of insemination in order to inhibit fertilization. Branham and Metz (1959), however, reported that *Arbacia* eggs were irreversibly inhibited after exposure to *Fucus* extract.

Several physical effects of FeInh(Fu) on eggs have been demonstrated by Wicklund (1954b) and Runnström and Hagström (1955). The former investigator demonstrated that exposure to the inhibitor resulted in a stiffening of the egg cortex. The latter investigators found that FeInh(Fu) prevented the swelling of the egg jelly layer which normally occurred when eggs were placed in sea water.

Lundblad (1954) found that two proteolytic enzymes extracted from echinoderm eggs were inhibited by FeInh(Fu). Esping (1957b) further investigated the effect of *Fucus* extracts on enzyme systems *in vitro* and reported inhibition of hyaluronidase, DNAase, alpha amylase and urease. She concluded that the inhibitor acted on a non-specific protein part of the enzyme.

The chemical nature of the fertilization inhibitor from *Fucus* is little known. Wicklund (1954a) recognized two kinds of inhibitors, one ("polysaccharide")

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which became inactive when oxidized by periodate, the other ("phenolic substance") which was not affected by this oxidizing agent. It was demonstrated, however, that the purified fucoidin preparations had no inhibitory effects (*cf.* Wicklund, 1954a). Esping (1957b) investigated the chemical nature of the hyaluronidase inhibitor for *Fucus* and concluded that it was probably a polyphenolic compound.

The present report is concerned primarily with further investigations of the effects of *Fucus* extracts on echinoderm spermatozoa. In contrast to the observations of Runnström and Hagström (1955), the *Fucus* extracts prepared in this laboratory prevented fertilizin agglutination of the sperm. The results presented here indicate that the sperm antifertilizin is inactivated by the agent(s) which is absorbed by the sperm. A second effect of the *Fucus* extract is to increase the rate of O_2 consumption of the spermatozoa. Despite these effects the sperm were capable of fertilizing eggs after exposure to the inhibitor. Eggs, however, fail to raise fertilization membranes or cleave when inseminated after exposure to the *Fucus* extract solutions.

Materials

Extracts of the alga *Fucus vesiculosus* were prepared by a modification of the method reported by Esping (1957a, method C). Fresh *Fucus*, obtained from the intertidal zone at Woods Hole, Mass., were repeatedly extracted by soaking the moist alga in 5 to 10 changes of tap water (Fig. 1). The extract thus obtained was concentrated under vacuum at temperatures below 80° C. Air was bubbled

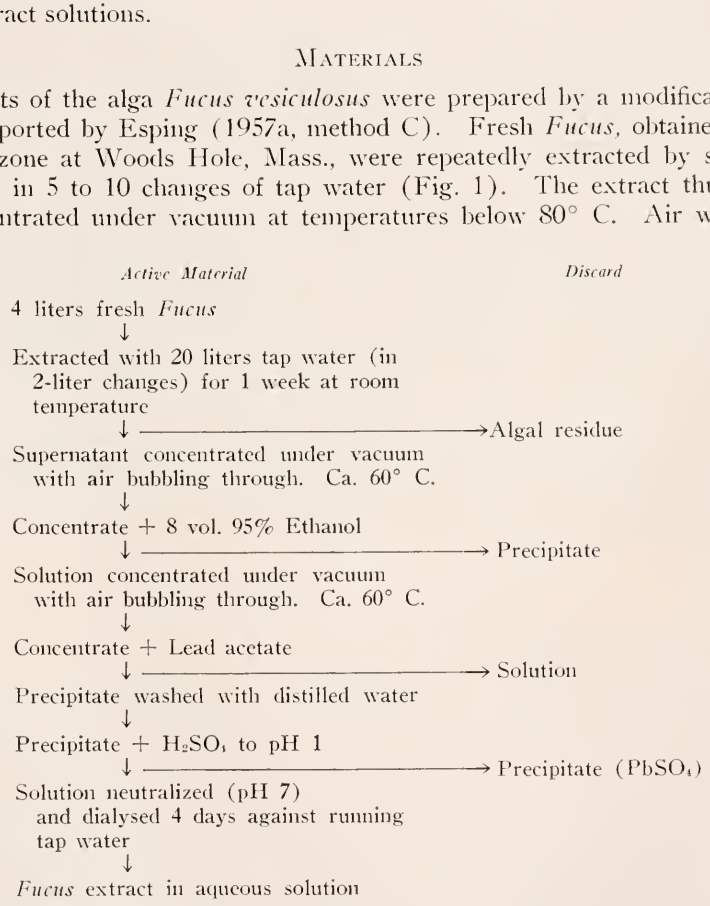

Figure 1. A method for the preparation of *Fucus* extract.

through the extracts to speed evaporation. The concentrated syrup was added to 8–10 volumes of 95% ethanol and the supernatant obtained after removal of the precipitate was again concentrated under vacuum. Saturated lead acetate was added to the ethanol-soluble fraction until precipitation was complete. The precipitate was collected and washed, then reacted with sulfuric acid. The precipitate (primarily lead sulfate) was removed and the supernatant neutralized with sodium hydroxide and dialysed against running sea water or tap water. In the latter instance isotonicity was obtained by mixing with an equal volume of sea water concentrated to half the original volume or diluting with more than 100 volumes of sea water just before use.

No precautions were taken to avoid bacterial contamination in the long extraction. However, upon repetition, the procedure gave consistent results. Furthermore, other methods, including extraction by boiling, gave preparations with similar properties. Therefore, it is likely that the active material is not a product of bacterial action.

The amount of dry matter in the extract solutions was determined by dialysing aliquots against distilled water and drying to constant weight at 85° C. It was difficult to redissolve the dry material and most of the activity of the preparation was not recoverable after drying. For this reason the *Fucus* extracts were kept in solution and the amount of dry matter used in the tests was calculated from the volume used.

All experiments except those dealing with specificity employed gametes of *Arbacia punctulata*. The animals were from the Woods Hole, Mass., area or from the Gulf of Mexico in the vicinity of the Florida State University Marine Laboratory at Alligator Point, Florida. Gametes were shed by the usual methods of electrical stimulation or the application of isotonic KCl to the gonads (Costello *et al.*, 1957). Sperm concentration was estimated by measuring the optical density of the sperm suspension with a Klett-Summerson colorimeter at 420 mμ. The standard curve was prepared by measuring the optical density of sperm suspensions and counting the spermatozoa in a Levy counting chamber (haemocytometer). Fertilizin was prepared by decanting the supernatant "egg water" from standing egg suspension, or by acid sea water (pH 5) extraction of the egg jelly material followed by neutralization with sodium hydroxide.

Experiments and Results

Inhibition of fertilizin agglutination of sperm

Runnström and Hagström (1955) reported that FeInh(Fu) did not agglutinate *Paracentrotus lividus* sperm or prevent sperm agglutination with homologous fertilizin. The *Fucus* extracts prepared in this laboratory were similarly tested with *Arbacia* sperm and fertilizin. One drop of dilute sperm (about 1% semen in sea water) was added to two drops of *Fucus* extract in two-fold dilution series. One drop of fertilizin was then added to the sperm mixture, and the agglutination observed macroscopically. Sperm in higher concentrations of the inhibitor remained motile but failed to agglutinate when fertilizin was added. In lower concentrations of the extract they agglutinated normally. The amount of extract necessary to inhibit fertilizin agglutination varied with different extracts. The

most active extract prepared inhibited agglutination at a concentration of 7.34×10^{-6} gm. of dry material/ml. when tested with 0.5% semen. Similar inhibition of fertilizin agglutination was observed with sperm and fertilizin from the two other species tested (*Mellita quinquiesperforata* and *Echinarachnius parma*). Thus the fertilizin-agglutination inhibiting action of the *Fucus* extract was not specific.

The *Fucus* extract caused several visible changes in the sperm. In the highest concentrations of the most potent inhibitor preparations sperm slowly agglutinated when diluted in sea water. In more dilute (but still inhibiting) concentrations of the extract, the sperm became more motile than control sperm in sea water.

The inhibition of fertilizin agglutination of sperm could have resulted from an action of *Fucus* extract on fertilizin, on the sperm, or both. To test the first of these possibilities attempts were made to restore agglutinating activity to non-agglutinating *Fucus* extract-fertilizin mixtures (*cf.* Metz, 1959). As seen in Table I (one of four similar experiments) treatment with activated charcoal ("Norite") restored the agglutinating activity of such a mixture to virtually control levels. Apparently, the charcoal differentially adsorbed the inhibiting agent without appreciable effect on the fertilizin (*cf.* Tyler and Fox, 1940). It is clear from this that the *Fucus* extract does not irreversibly destroy the sperm agglutinating action of fertilizin. This view is supported also by the observation (Table I) that dilution of the non-agglutinating mixture with sea water restores agglutinating action. These observations suggest that the *Fucus* extract does not affect the fertilizin but instead acts upon the sperm.

To test more directly whether the inhibition resulted from an effect on sperm, the following experiment was performed. Sperm were mixed with *Fucus* extract to produce a suspension which did not agglutinate with fertilizin. Then the sperm were centrifuged down, the supernatant replaced with sea water and the sperm resuspended. This washing was repeated several times and the sperm tested for

TABLE I*

Agglutinating capacity of Arbacia fertilizin after exposure to Fucus extract

Dilution of mixture	Fertilizin + *Fucus* extract		Fertilizin + sea water	
	Absorbed with Norite	Unabsorbed	Absorbed with Norite	Unabsorbed
1/2	++++	−	++++	++++
1/4	++++	++	++++	++++
1/8	++++	++++	++++	++++
1/16	++++	++++	++	++++
1/32	+++	+++	+	+++
1/64	++	++	−	++
1/128	±	+	−	+

* One ml. of fertilizin was mixed with one ml. of *Fucus* extract (dialysed against sea water) or with sea water. One ml. of activated charcoal ("Norite") was then added to each and removed by filtration. The mixtures were tested for agglutinating action before and after absorption with charcoal. To test for agglutinating action one-drop aliquots of the filtrates were serially diluted in two-fold steps and mixed with one-drop samples of semen diluted to 1% with sea water. The results are recorded as agglutinations.

TABLE II*

Fertilization agglutination of Arbacia sperm after washing from Fucus extract

Times centrifuged	Sperm + *Fucus* extract centrifuged		Sperm + sea water centrifuged	
	Fertilizin agglutination of sperm after resuspension in sea water	Fertilizin agglutination of control sperm in supernatant	Fertilizin agglutination of sperm after resuspension in sea water	Fertilizin agglutination of control sperm in supernatant
1	−	−	++++	++++
2	++	++++	++++	++++
3	+++		++++	
4	++++		++++	

* Two-ml. samples of 5% semen were mixed with 2 ml. of *Fucus* extract (dialysed against sea water) or sea water. After 32 minutes both samples were centrifuged (4° C.), the supernatants were withdrawn and the sperm resuspended to 4 ml. in sea water. One-drop aliquots of sperm suspension were tested for agglutination by adding one drop of fertilizin. The centrifugation of the main sample was then repeated. One-drop aliquots of the supernatants were tested for excess inhibitor by adding one drop of 1% control sperm, then one drop of fertilizin. The results are recorded as agglutination.

agglutination with fertilizin after each washing. One of the six experiments performed appears in Table II. In all experiments the sperm failed to agglutinate after the first resuspension in the sea water, even though the wash water (the supernatant from the second centrifugation) did not inhibit when tested with control sperm. With repeated washing the spermatozoa regained some ability to agglutinate when mixed with fertilizin. The number of washings required to restore agglutinability to the sperm varied somewhat. In one experiment treated sperm did not agglutinate with fertilizin until after five washings. This experiment was run in parallel with fertilizin-treated reversed sperm, which also recovered agglutinability after five washings. Repeated washing altered the fertilizin

TABLE III*

Agglutination titer of fertilizin after exposure to Fucus extract-treated sperm.

Supernatant dilution	*Fucus* extract-treated sperm + fertilizin	Sea water-treated sperm + fertilizin	*Fucus* extract + fertilizin	Sea water + fertilizin
1/2	++++	++++	−	++++
1/4	++++	+++	−	++++
1/8	++++	++	−	++++
1/16	+++	+	+	++++
1/32	++	−	+	+++
1/64	+	−	+	++
1/128	−	−	+	+

* One ml. of packed sperm (or sea water) was mixed with one ml. of *Fucus* extract (or sea water) and centrifuged down (4° C.). The sperm (or one ml. of solution) were resuspended in two ml. of fertilizin and recentrifuged. One-drop aliquots of the supernatants were diluted in two-fold steps and mixed with one drop of 0.5% sperm. The results are recorded as agglutination.

TABLE IV*

Absorption of Fucus extract color and activity by Arbacia sperm

	Fucus extract + sea water	*Fucus* extract + sperm	Sea water + sperm	Sea water + sea water
O.D.	.86	.37	.06	.00
Fertilizin agglutination inhibition titer	6	0	0	0
Fertilization inhibition titer	8	2	0	0

* Five ml. of *Fucus* extract (1.0×10^{-4} gm./ml.) or sea water were mixed with two ml. of washed sperm or sea water and after five minutes centrifuged. Optical density of the resulting supernatant was measured with a Klett-Summerson colorimeter (420 μm.). Fertilizin agglutination inhibition titer is the reciprocal of the last dilution (in a two-fold dilution series) in which no agglutination occurred when one drop of inhibitor was mixed with one drop of 0.5% semen. Fertilization inhibition titer is the reciprocal of the last dilution (two-fold) in which 1% or less cleavage occurred after one drop of eggs was added and inseminated with two drops of 10^{-3} semen.

agglutination of sperm. The agglutinates formed more slowly than with unwashed sperm, and also reversed more slowly, even though the spermatozoa remained motile. This effect was more pronounced on the inhibitor-treated sperm than on sperm treated in sea water.

The above experiments indicate that the *Fucus* extract acted upon the sperm to inhibit agglutinability with fertilizin. Additional evidence for this was found in experiments demonstrating that the inhibitor-treated, non-agglutinating sperm failed to absorb fertilizin as readily as did control sperm (Table III) (*cf.* Lillie, 1914). In two experiments inhibitor-treated and control sperm were added to fertilizin, centrifuged out, and the supernatant titrated for agglutinating activity. In both experiments the control sperm reduced the agglutinating activity substantially more than inhibitor-treated sperm. It is concluded from these experiments that the *Fucus* extract inactivated the specific fertilizin receptor sites of the sperm surface (antifertilizin) to render them unreactive to fertilizin. "Antifertilizin" extracted from sperm by the freeze-thaw method of Tyler (1939) lost its egg jelly coat precipitating activity after mixing with *Fucus* extracts, although dilution controls remained active. A precipitate formed when the "antifertilizin" preparation was mixed with *Fucus* extract.

Sperm appeared to remove the brown color from *Fucus* extract solution. This was confirmed by measuring the optical density of a *Fucus* extract solution with a Klett-Summerson colorimeter (blue filter, 420 mμ.) before and after absorption with sperm (Table IV). The solution was also titrated for inhibitory activity after absorption with sperm. In five experiments sperm decreased the optical density of *Fucus* extract solutions and removed or greatly reduced both the fertilizin agglutination and the fertilization inhibiting properties of the solutions. The sperm used in the absorption became more darkly colored than control sperm. Thus, it appears that sperm reacted with and removed from solution the color and active substance(s) of the *Fucus* extract, and that concomitantly the sperm lost the ability to react with fertilizin from the egg.

Sperm motility and oxygen consumption in Fucus extracts

Wicklund (1954c) reported that sperm were more active in FeInh(Fu) than in sea water. The extracts prepared in this laboratory also stimulated the motility of spermatozoa. However, in high concentrations of the more potent inhibiting preparations, sperm became immotile more quickly than sperm in sea water.

The stimulation of sperm was also examined in terms of oxygen consumption measured manometrically with the Warburg apparatus. In four experiments the rate of oxygen consumption increased dramatically upon the addition of *Fucus* extract to the sperm (Fig. 2). In the experiments represented in the figure the

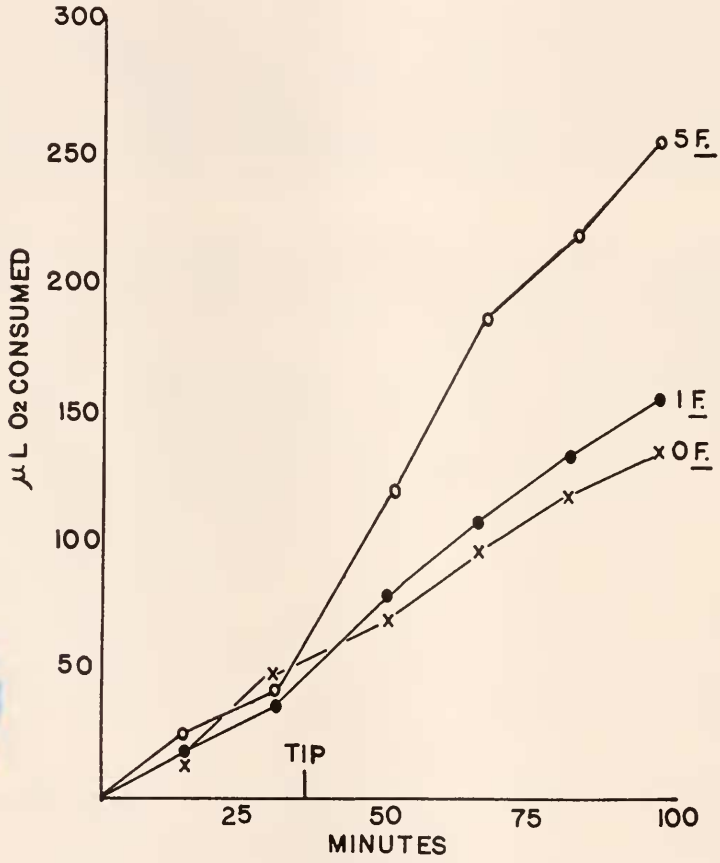

FIGURE 2. Effects of *Fucus* extracts on the oxygen consumption of sperm. Curve 5F (open circles) 5.0×10^{-4} gm. of Fucus extract per ml.; Curve 1F (solid circles) 1.0×10^{-4} gm. of *Fucus* extract per ml.; 0F (crosses) sea water control containing no *Fucus* extract. Oxygen consumption was measured with a standard Warburg apparatus. Experiments were performed in duplicate in 15–20-ml. single side arm flasks at 20° C. The vessels were shaken at 120 cycles/min, with an amplitude of 3.5 cm. Vessels contained 1.8 ml. of washed sperm (2.0×10^{10} sperm/ml.). Two-tenths ml. of 10% KOH was in the center well. One ml. of sea water or *Fucus* extract was tipped into the vessels at 35 minutes. All solutions were buffered to pH 8.0 with 0.02 *M* glycylglycine.

pH was controlled by using sea water buffered with .02 *M* glycylglycine (Tyler and Horowitz, 1937). At the beginning of the experiment sea water, sperm and *Fucus* extract were adjusted to pH 8.0. At the end of the experiment, one hour after tipping the test substance, the pH was again measured and had dropped to pH 7.8 in the most actively respiring systems.

The observed increase in oxygen consumption was clearly the result of enhanced sperm respiration, not a direct oxidation of the *Fucus* extract. This was shown in three experiments in which *Fucus* extract did not consume oxygen when tipped into sea water.

It will be seen from Figure 2 that the rate of O_2 consumption increased upon addition of *Fucus* extract. The rate was maximum directly after tipping and then decreased. No dilution effect was observed in the sea water control. The change in rate of oxygen consumption was related to the concentration of the *Fucus* extract. A maximum rate (0–15 minutes after tipping) of 6.4×10^{-9} μl. O_2/hr./sperm resulted when 5.0×10^{-4} gm. of extract was added to 3.6×10^{10} sperm (or 1.4×10^{-14} gm./sperm). This represents a 2.8-fold increase over the control rate of 2.3×10^{-9} μl. O_2/hr./sperm. When 1.0×10^{-4} gm. of extract was added to the same amount of sperm (or 0.28×10^{-14} gm./sperm) only a 1.3-fold increase in rate was observed (to 3.1×10^{-9} μl. O_2/hr./sperm). In a separate experiment, employing a one-tenth dilution of the same sperm used in the experiment in Figure 2 (3.4×10^9 sperm), the addition of 1.0×10^{-4} gm. of extract (or 2.9×10^{-14} gm./sperm) resulted in a 6.2-fold increase in rate (from 8.2×10^{-9} to 51×10^{-9} μl. O_2/hr./sperm).

Fertilizing capacity of Fucus extract-treated sperm

Inhibition of fertilization apparently does not result from an irreversible action on spermatozoa. Wicklund (1954a) reported that sperm suspended in FeInh(Fu) were capable of fertilizing eggs if the inhibitor was diluted out during insemination. The *Fucus* extracts prepared in this laboratory were tested for effects on the fertilizing capacity of *Arbacia* sperm. The results obtained were basically the same as reported by Wicklund. That is, treated sperm were capable of fertilizing eggs. However, in 8 of 22 experiments the fertilizing capacity of treated sperm was reduced, in the sense that more treated than control sperm were required to achieve the same percentage of cleaved eggs.

To test for effects on the fertilizing capacity, *Arbacia* sperm were suspended in *Fucus* extract solution, then centrifuged down and resuspended in sea water. The washed sperm were then serially diluted and used to inseminate eggs. The supernatant removed after centrifugation was tested for an excess of fertilization inhibitor by adding eggs and inseminating with control sperm. The agglutinating capacity of the treated sperm was tested by adding a drop of fertilizin to a drop of washed sperm. Only those experiments wherein the treated sperm failed to agglutinate are considered. The number of sperm was determined turbidimetrically after washing to be sure that the amounts of treated and control sperm were equal.

In 14 experiments sperm treated in *Fucus* extracts were as successful in activating eggs as sperm treated in sea water. In 8 experiments, however, more *Fucus* extracted-treated sperm than control sperm were required to achieve equivalent percentages of cleaved eggs. One of these experiments is presented in Table V.

TABLE V*

The fertilizing capacity of Fucus extract-treated sperm

Sperm/ml.	*Fucus* extract-treated sperm 4 ml. + 1 drop eggs	Sea water-treated sperm 4 ml. + 1 drop eggs
6.4×10^9	100% cleaved	100% cleaved
1.3×10^9	99%	100%
2.6×10^8	9%	86%
5.4×10^7	5%	55%
1.1×10^7	1%	2%
2.2×10^6	0	1%
Inhibitor solution after centrifugation	0	100%

* Two ml. of diluted *Arbacia* sperm were mixed with two ml. of *Fucus* extract (2.5×10^{-5} gm./ml.) or with sea water. The suspension was centrifuged in the cold and the sperm resuspended in 10 ml. of sea water. The sperm concentration was determined turbidimetrically and the suspension diluted in five-fold steps. One drop of eggs was added to four ml. of the sperm. Eggs were also added to the supernatant (solution after treatment of sperm) and inseminated with one drop of 3.2×10^{10} sperm/ml. Results are presented as percentage of cleaved eggs (100 eggs counted).

In all eight of the experiments wherein reduced fertilizing capacity was observed, sperm were treated with samples from a single very "active" inhibitor preparation (100% inhibition of cleavage in 1.88×10^{-6} gm. of *Fucus* substance/ml.). The highest percentage of cleavage obtained in each of these eight experiments occurred in the highest concentrations of sperm and consequently the highest concentrations of any residual inhibitor. In four experiments the same inhibitor did not reduce the fertilizing capacity of sperm, although an excess of inhibitor was demonstrated in the first supernatant (treating solution) and the treated sperm failed to agglutinate when added to fertilizin solution.

Fertilizability of Fucus extract-treated eggs

Wicklund (1954a) reported that the effect of FeInh(Fu) on fertilization was reversible. Eggs treated with the *Fucus* extracts prepared in this laboratory, however, failed to recover fertilizability after repeated washing with sea water, or digestion by enzymes. This inhibition was not the result of "killing" the eggs, for eggs fertilized in sea water and then placed in otherwise inhibitory solutions of *Fucus* extracts cleaved normally.

Eggs were exposed to *Fucus* extract solution, extensively washed in sea water and then inseminated. The results of one of nine similar experiments are presented in Table VI. It is clearly seen that eggs treated with the extracts are not fertilizable even after repeated washing with sea water. In the nine experiments some of the eggs were also passed through (one hour) 1% solutions of trypsin, lysozyme, pectinase or pectinesterase. Control eggs remained fertilizable after exposure to the protein solutions but eggs exposed to *Fucus* extract, before digestion with the above enzymes, failed to cleave after insemination.

This irreversible inhibition of fertilization is apparently not due to general cytotoxic effects on eggs. Eggs placed in fertilization-inhibiting concentrations of

TABLE VI*

Fertilizability of Fucus extract-treated eggs

Sperm dilution	*Fucus* extract-treated eggs washed in sea water	Sea water-treated eggs washed in sea water
1	0 cleaved	95% cleaved
1/5	0	95%
1/25	0	100%
1/125	0	92%
1/625	0	63%

* Ten drops of *Arbacia* eggs were placed in five ml. of sea water or *Fucus* extract ($2.5 \times 10^{-}$ gm./ml.) for five minutes. The eggs were then washed through 8 five-ml. changes of sea water over a period of 90 minutes. One drop of eggs was added to four ml. of serially diluted sperm and the results reported as per cent cleavage (100 eggs counted).

extract five minutes after insemination cleaved synchronously with untreated controls if left in the inhibitor or washed out of it. Also, in three experiments eggs predigested with trypsin were not inhibited by exposure to *Fucus* extract. These experiments indicate that the *Fucus* extract solutions do not kill eggs or inhibit the cleavage process, but do interfere with or block an initial stage(s) of fertilization.

Effects of tannic acid on Arbacia gametes

Similarities between the effects of *Fucus* extracts and tannic acid on *in vitro* enzyme systems were pointed out by Esping (1957b). Kylin (1938) found that brown algae contained substances similar to tannins. Thus it seemed of interest to examine the effects of tannic acid on sea urchin gametes and fertilization (*cf.* Branham and Metz, 1960). It was found that tannic acid affected sperm in a manner similar to *Fucus* extracts and inhibited fertilization by an irreversible action on eggs.

Baker (C.P.) tannic acid was found to inhibit fertilizin agglutination of sperm (*Arbacia*) in concentrations of about 3.2×10^{-3} gm./ml. Sperm remained motile at this dilution. In three experiments sperm treated with tannic acid, centrifuged down and resuspended in sea water failed to agglutinate when tested with fertilizin, even though the supernatant from a second centrifugation (the wash water) was free of inhibitory activity when tested on control sperm. Repeated washing restored some agglutinability to the treated sperm but the results of these experiments were equivocal in that such repeatedly washed sperm spontaneously agglutinated upon dilution in sea water.

Tannic acid also inhibited fertilization. Complete inhibition of cleavage resulted when eggs were inseminated in concentrations of 6.0×10^{-5} gm. of tannic acid/ml. or greater. Washing tannic acid-treated eggs in sea water failed to restore fertilizability. The motility of sperm was not impaired in inhibiting concentrations of tannic acid, and eggs placed in the inhibiting solution 5 minutes after insemination cleaved normally. Thus tannic acid and *Fucus* extracts both inhibit fertilization by an irreversible action on eggs.

It was also found that the inhibitory titer of tannic acid increased upon oxidation. In one experiment 1% tannic acid in distilled water was raised to pH 10 with NaOH and air was bubbled through the solution for one week. The preparation was then dialysed against distilled water and tested for inhibitory effects. This treat-

ment increased the capacity of tannic acid to inhibit fertilizin agglutination of sperm by about 10-fold (from 3.2×10^{-3} gm./ml. to 2.2×10^{-4} gm./ml. for complete inhibition), but the fertilization inhibition titer remained essentially the same as before the oxidation.

Discussion

The experiments presented here demonstrate several effects of extracts of the brown alga *Fucus vesiculosus* upon sea urchin gametes. These include stimulation of motility and increase in rate of oxygen consumption of spermatozoa, inhibition of agglutination of sperm by fertilizin and finally inhibition of fertilization.

The inhibition of fertilizin agglutination of sperm results from the action of the inhibiting preparation on the sperm to render these cells unagglutinable to fertilizin. Evidence for such action was obtained in washing experiments. Sperm treated with inhibiting concentrations of *Fucus* extract failed to regain agglutinability upon washing in sea water. Repeated washings, however, do restore agglutinability to treated sperm. To this extent the effect of the inhibitor is reversible. The *Fucus* extracts not only inhibited agglutination but actually rendered sperm incapable of combining with fertilizin. This action was demonstrated by failure of treated sperm to absorb fertilizin. The *Fucus* preparation failed to irreversibly inactivate fertilizin, as shown by restoration of agglutinating activity to non-agglutinating fertilizin-*Fucus* extract mixtures by absorption with charcoal.

One explanation for the observed inhibition of fertilizin agglutination is that inhibitory material in the *Fucus* extracts combined with the sperm surface to inactivate the antifertilizin. The observation that sperm removed the inhibitor from solution is additional evidence for such a mechanism. The inactivation could result from direct combination of the inhibitor with the active site or from a steric masking of the antifertilizin. The recovery of agglutinability upon repeated washing can be explained by removal of the masking substance or by stripping off of the inactivated antifertilizin-inhibitor complex to expose unreacted sites.

Another explanation is that the inhibitor removed antifertilizin from the sperm surface. The gradual recovery of agglutinability with repeated washing is evidence that this is not the case. If it is assumed that the recovered agglutinability is due to the same kind of reactive site as normal agglutination, it would be expected that the effect of the inhibitor would disappear as soon as the sperm were washed. The observed slow appearance of agglutinability after repeated washing is not accounted for by this hypothesis.

Esping (1957b) suggested that *Fucus* extracts denature proteins in the same way as tannic acid. The observation of Wicklund (1954a) that albumin and some other proteins reverse the effect of FeInh(Fu) on eggs could also be explained by a tanning reaction between the inhibitor and the protein resulting in inactivation or removal of the inhibitor. The similarities reported here between the action of *Fucus* extracts and tannic acid suggest that the inhibitory effects might be due to a tanning action. The inhibition of fertilizin agglutination could be explained by hypothesizing precipitation of sperm surface proteins by depsides (aromatic tannagins).

If *Fucus* extracts are assumed to contain depside-like characteristics, then the failure of the inhibitor to destroy the agglutinating properties of fertilizin is of some

interest. Fertilizin apparently is an acid mucopolysaccharide which is not readily separated into distinct protein and carbohydrate moieties (Metz, 1957). At least one way that depsides react with proteins is through amino groups of the protein by hydrogen bonding or ionic linkage with anionic sites on the tannic acid (Gustavson, 1956). Failure of tannic acid to inactivate fertilizin, then, indicates that such cationic groups are not essential for agglutinating activity. This conclusion agrees with results obtained previously using protein group reagents (Metz, 1954).

The stimulation of motility and the increased rate of O_2 consumption that occurs when spermatozoa are mixed with *Fucus* extracts are striking but shed little light on the mechanism of inhibitor action. Numerous agents cause similar effects (*cf.* Rothschild, 1956). In experiments with *Fucus* extracts, sperm at first become hyperactive but later immotile. The rate of respiration remained high even after the sperm became immotile. This suggests that the *Fucus* substances could uncouple oxidative phosphorylation.

Despite the effects of *Fucus* extracts on fertilizin agglutination and respiration, sperm treated with the extracts are capable of fertilizing eggs. In 14 of 22 experiments the fertilizing capacity of treated sperm was the same as that of control sperm, even though the treated sperm did not agglutinate with fertilizin. This observation is in agreement with the report of Tyler and Metz (1955) that fertilizin-treated *Arbacia* sperm showed no reduction of fertilizing capacity when tested on normal eggs. These experiments also indicate that the *Fucus* extracts had no deleterious effects upon the egg-activating properties of spermatozoa. Thus sperm motility, acrosome reaction and surface groups that can be postulated to have a role in egg activation must not have been impaired by exposure to the fertilization inhibitor from *Fucus* in these 14 experiments. In 8 other experiments the fertilizing capacity of sperm was reduced upon exposure to *Fucus* extracts. The nature of this reduction has not been explained but it is interesting that it was not absolute. If enough treated sperm were present, eggs were activated. This indicates either that many partially inhibited sperm could combine forces to bring about egg activation or that some sperm were not inactivated at all. The all-or-none hypothesis seems more reasonable because the resulting cleavages were normal, that is to say, into two equal blastomeres, indicating reaction with a single spermatozoan. In all 22 experiments the treated sperm were capable of fertilizing eggs, and thus an explanation of the action of the fertilization inhibitor from *Fucus* must be sought elsewhere.

The inhibition of fertilization results from an irreversible action upon eggs. This is clearly demonstrated in numerous experiments wherein eggs were washed repeatedly in sea water following treatment in *Fucus* extracts but remained unfertilizable. This irreversible inhibition apparently does not involve general cytotoxic effects upon eggs. This is demonstrated by the normal cleavage of eggs inseminated in sea water and then placed in otherwise inhibiting concentration of the *Fucus* extracts. Eggs pretreated with trypsin and subsequently exposed to the extracts, fertilized upon insemination, again demonstrating that the *Fucus* extracts apparently have no general toxic action on the egg. Fertilization inhibition apparently involves inactivation of some initial step(s) of fertilization of the egg, perhaps by a depside-like reaction with the egg surface. In further studies attempts will be made to identify the site of such action.

Summary

1. Extracts of the brown alga *Fucus vesiculosus* inhibit fertilization and the fertilizin agglutination of *Arbacia* sperm.

2. The inhibition of fertilizin agglutination apparently results from an action on sperm. Sperm washed from the extracts do not agglutinate. Furthermore, fertilizin is not irreversibly inactivated by *Fucus* extracts. The color and inhibitory properties of *Fucus* extracts are removed from solution by absorption with sperm.

3. Sperm motility and O_2 consumption were stimulated by *Fucus* extracts.

4. The inhibition of fertilization is the result of an irreversible action on eggs. The fertilizing capacity of sperm, however, is not destroyed by exposure to inhibitory amounts of *Fucus* extract.

5. Tannic acid showed inhibitory properties similar to the *Fucus* extracts. It inhibited fertilization by an irreversible effect on eggs and prevented fertilizin agglutination by an effect on sperm. Tannic acid-treated sperm were capable of fertilizing eggs.

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